

Serum specific-IgE antibodies to peptides detected in a casein hydrolysate formula

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Up to 2.5% of infants are affected by cow milk hypersensitivity in the first two years of life, although most of these children will “outgrow” their reactivity within 2–3 years. Extensively hydrolyzed (“hypoallergenic”) cow milk-based formulae are often recommended as a substitute formula and are generally well tolerated. However, a small minority of cow milk-allergic children experience allergic reactions to the hypoallergenic formulae as well. Utilizing inhibition-ELISAs and sera from patients with IgE-mediated cow milk allergy, we have identified residual protein fractions less than 20 kD in several of the extensively hydrolyzed cow milk-based formulae. Although many of the cow milk allergic children had positive skin prick tests [SPT] to one of the hydrolysate formulae (Nutramigen™), the positive skin test result generally did not correlate with clinical reactivity, although the negative predictive value of the negative SPT was excellent. Children with IgE-mediated cow milk allergy and a positive skin prick test to the hypoallergenic formula should probably receive their first dose of the formula in a medical setting so that appropriate therapy can be administered in the unlikely event of an allergic reaction.

**Kristina M. Hoffman¹,
Hugh A. Sampson²**

Johns Hopkins University School of Medicine,
Division of Pediatric Allergy and Immunology,
Baltimore, Maryland, USA

Hugh A. Sampson, Mount Sinai School of
Medicine, Department of Pediatrics Box 1198,
One Gustave L. Levy Place, New York,
NY 10029-6574, USA
Telephone: (212) 241-5548,
Fax: (212) 828-4182

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Cow milk hypersensitivity is a significant problem in early childhood. Four prospective studies indicate that cow milk allergy affects about 2.5% of infants in the first 2 years of life (1–4). Extensively hydrolyzed cow milk-based formulas and soy protein-based formulas are often substituted in children with symptoms of cow milk allergy. However, our recent multi-center study indicated that about 15% of cow milk allergic children also are sensitive to soy-based formulae (5). In addition, about 50% of infants with the cow milk-induced enterocolitis syndrome will develop soy protein-induced enterocolitis if soy is substituted as a protein source at the time of diagnosis (6). Rare patients with IgE-mediated cow milk allergy who react to extensively hydrolyzed (“hypoallergenic”) formulae have been reported in some cow milk allergic patients (7–13). Although positive RAST-inhibitions to “hypoallergenic” formulae have been reported with sera from cow milk allergic children (8, 12, 14), the detection of serum IgE antibodies to specific protein fractions found in an extensively hydrolyzed casein formula have not been

reported previously.

Recently Dean and co-workers reported the presence of low molecular weight proteins in Nutramigen and other extensively hydrolyzed milk-based formulae using tricine-SDS PAGE (15). In the present study, we have identified residual protein fractions in extensively hydrolyzed cow-milk-based formulas using tricine-SDS gel electrophoresis and inhibition ELISA. Immunoblot analyses to one of the formulas, Nutramigen (Mead Johnson), were performed using sera from 15 cow milk allergic children with positive skin prick tests (SPTs) to cow milk and positive double-blind placebo controlled food challenges (DBPCFCs) or a definitive clinical history of anaphylactic symptoms to milk. Five of these patients had positive oral challenges to Nutramigen and positive SPTs to Nutramigen, five had positive SPTs to Nutramigen, but no clinical reactivity to Nutramigen, and five had both negative SPTs to Nutramigen and no clinical reactivity to Nutramigen. The presence of serum specific-IgE antibodies to the protein fractions in Nutramigen in these three groups of cow milk allergic patients was compared with their clinical reactivity upon ingestion of the formula.

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Methods

Infant formulae and milk proteins. Nutramigen (Mead Johnson, Bristol Meyer, Evansville, IN) and Alimentum (Ross Laboratories, Columbus, OH) were purchased retail. Profylac (ALK) and Alfa-Re (Nestle) were supplied by their European manufacturers since they are not commercially available in the U. S. A. The individual milk proteins were purchased from Sigma Chemical Co. (St. Louis, MO).

Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Schagger and von Jagow (16). The infant formulas and protein samples were incubated for 30 minutes at 40°C in 4% SDS, 12% glycerol, 50 mM Tris, 2% 2-mercaptoethanol, and 0.01% bromphenol blue, at a pH of 6.8. The sample load for the infant formulae was 1.9 mg of powdered formula per lane, and for the individual milk proteins was 5 mcg of protein per lane. Electrophoresis was performed at 4°C, initially at 30 V constant for 1 hour and then at 85 V constant for 18 hours.

Silver stain technique. Following electrophoresis the gel was fixed in 50% methanol for 2 hours, and then rinsed and soaked in deionized H₂O (dH₂O) for 30 minutes. The staining solution was prepared as follows: 42 ml of 0.36% NaOH was mixed with 2.8 ml of NH₄OH. Six milliliters of a 20% AgNO₃ was added dropwise to the first solution and then the total volume was increased to 200 ml with dH₂O. The gel was submerged in this solution for 15 minutes in the dark, and then washed for 5 minutes in dH₂O. The developing solution was prepared as follows: 2.5 ml of 1% citric acid and 0.25 ml of 38% formaldehyde were mixed and increased to a total volume of 500 ml with dH₂O. The gel was developed in this solution until dark protein bands appeared, and then the gel was removed and washed for 5 minutes in dH₂O (17).

Patients characteristics, skin prick tests, food challenges, and serum collection. Patients in this study ranged in age from <1 year to 15 years. Skin prick tests were performed with undiluted, ready-to-feed Nutramigen, cow milk extract, (1: 10 w/v; Greer Laboratories, Lenoir, NC), histamine control (1 mg/n-ml), and saline control as previously described (18). Skin prick tests were considered positive if the mean wheal diameter was 3 mm or greater than the negative (saline) control. All 15 patients had positive skin prick tests (SPTs) to cow milk and were found to be cow milk allergic by double-blind placebo controlled food challenges as previously de-

scribed (19) or by a definitive clinical history of anaphylactic symptoms after the isolated ingestion of cow milk. There were five patients in each of the following groups:

Group #1 – positive SPT to Nutramigen and clinical reactivity to Nutramigen;

Group #2 – positive SPT to Nutramigen and no clinical reactivity to Nutramigen;

Group #3 – negative SPTs to Nutramigen and no clinical reactivity to Nutramigen.

Venous blood was collected from each patient and the serum separated and frozen at -20°C until used in the immunoblot and enzyme-linked immunosorbent assays.

Immunoblots. Following tricine-SDS PAGE, separated protein fractions were transferred electrophoretically to nitrocellulose paper and blocked overnight with PBS-Tween and 0.5% gelatin and washed with PBS. Nitrocellulose strips were incubated with patient sera at a 1:5 dilution for 3 hours and then underwent five 5 min washes with PBS-Tween. The nitrocellulose strips were then incubated with phosphatase labeled goat anti-human IgE antibody (Kirkgaard Perry Laboratories; Gaithersburg, MD) for 2 hours, washed, and developed with 5-bromo-4-chloro-indolyl phosphate/nitro blue tetrazolium.

Controls included a strip where no patient sera was added in the initial incubation in order to rule-out non-specific binding by the detection antibody. Inhibition analyses were performed on selected serum samples to demonstrate specificity of binding; Nutramigen was added to the serum sample during the initial incubation with the immunoblot strip, and then developed as described above.

Inhibition-enzyme-linked immunosorbent assays (Inhibition-ELISAs). Flat-bottomed 96well microtiter plates (Immunolon II, Dynatech Laboratories, Inc., Chantilly, VA) were coated overnight with 10 ng per well of casein or whey in 0.05 M carbonate buffer (pH 9.6). After appropriate washing, the plates were blocked with 0.2 ml of PBS containing 0.07% ovalbumin for one hour. Two-hundred microliters of each formula test sample were mixed with 200 ul of rabbit casein- or whey-antisera diluted 1: 150,000 in PBS containing 0.5% ovalbumin and 0.1% Tween-20, and incubated for 2 hours at 37°C.

One hundred microliters of the test-sample mixture (antigen-antibody complex) were then pipetted into individual wells of the coated microtiter plates and incubated for one hour at room temperature. The plates were washed 5 times in PBS-Tween, and horseradish peroxidase-labeled goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) per ml in PBS-Tween with 1 g of ovalbumin was added and incubated for 1 hour at room temperature. The mi-

croter plates were washed again in PBS Tween and developed with 3,3',5,5'-tetramethylbenzidine for 10 minutes at room temperature. The reaction was stopped by adding 0.6 M phosphoric acid, and the absorbance at 450 nm was measured (Molecular Devices, Menlo Park, CA).

Standard curves were generated by assaying known amounts of casein and whey in the same assay system. The percent absorbance at 450 nm was plotted against the protein concentration, and the linear portion of each curve was used in calculating the concentration of milk proteins in the test samples.

Table 1. ELISA results

	Casein (Ng/MI)	Whey (Ng/MI)
Nutramigen	414	911
Alimentum	1452	1478
Profylac	1747	3029
Alfa-Re	3139	14408
Cow milk	24.8×10^6	30.0×10^6

Results

Distinct protein fractions at 20 kD and 18 kD were detected in Nutramigen by tricine-SDS polyacrylamide electrophoresis and subsequent silver stain (Fig. 1). Diffuse protein staining was seen with Profylac at < 15 kD and with Alimentum at < 14 kD. No protein fractions were detectable in Alfa-Re utilizing this method.

Inhibition-ELISA results are depicted in Table 1. Nutramigen had the least detectable casein and whey contents with this assay. Of the four formulae tested, Alfa-Re had the highest detectable levels of casein and whey. The casein and whey content of

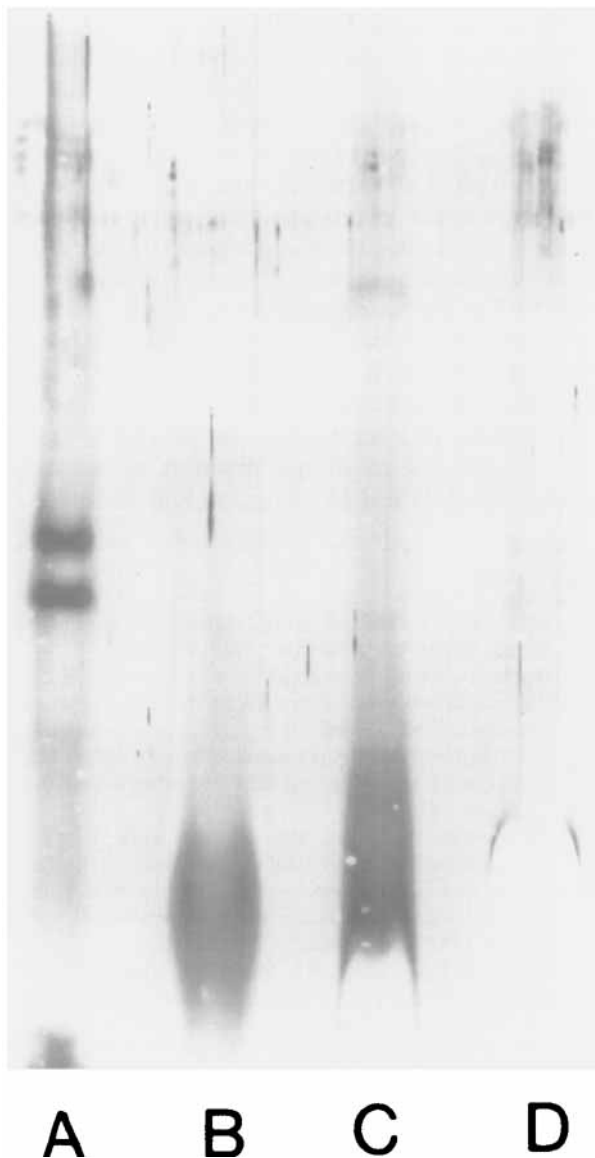


Fig. 1. Silver stain following protein fraction separation by tricine-SDS PAGE: Lane A: Nutramigen; Lane B: Alimentum; Lane C: Profylac; and Lane D: Alfa-Re.

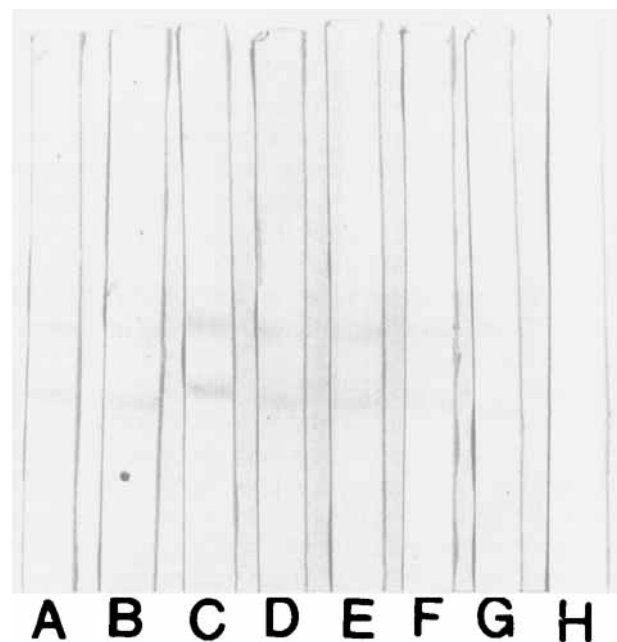


Fig. 2. Representative samples of immunoblot analyses.

A-D: Patients from Group One.

E: Patient from Group Two.

F: Patient from Group Three.

G: Control (no patient sera) revealed nonspecific binding of the goat anti-human IgE antibody to the lower molecular weight band of Nutramigen.

H: Inhibition of binding of patient C's specific IgE antibody to Nutramigen.

cow milk was at least 1000-fold higher than any of these formulae.

Immunoblot analyses to the 2 protein fractions detected in Nutramigen revealed specific IgE antibody to these proteins in 12 of 15 patients, four patients from each group. Representative immunoblots from each patient group are shown in Fig. 2. There was evidence of non-specific binding in the control immunoblot as indicated by binding of the goat anti-IgE antibody binding to the 18 kD fraction. The presence of serum specific IgE antibody to the protein fractions in Nutramigen did not correlate with clinical reactivity or positive SPTs to Nutramigen.

Discussion

In most cow milk allergic infants extensively hydrolyzed casein formulae do not provoke clinical symptoms (7). In our highly selected referral population, 149 children have been identified with IgE-mediated cow milk allergy; 14 (10%) were found to react to Nutramigen™ and/or Alimentum™ (six diagnosed by physician-supervised challenge and eight by "convincing" history of a reaction following an isolated ingestion). Allergic reactions to extensively hydrolyzed cow milk protein formulae in highly sensitive milk allergic children have been reported (7–13). In these reports, all patients experiencing clinical reactivity to a hydrolysate formula had a positive SPT to the hydrolysate formula. Conversely, only a small fraction of cow milk allergic patients with positive SPTs to a hydrolysate will react clinically to the formula. In a previous study, 11 of 25 milk allergic patients skin-tested to Nutramigen had a positive SPT result, but only two of these patients experienced a clinical reaction when orally challenged (7). Such findings are consistent with SPT results to whole proteins and clearly indicate that SPTs are not diagnostic of reactivity to hypoallergenic formulae.

Utilizing tricine-SDS-PAGE, Dean et al. were able to detect low molecular weight proteins in several extensively hydrolyzed milk protein-based formulae (15). In addition, previous reports of positive SPTs (7–13) and RASTs (8, 12, 14) to hydrolysate formulae in cow milk allergic children suggested the presence of allergenic peptides contained in these formulae. In this study, we were able to demonstrate the allergenic property of small protein fractions contained in an extensively hydrolyzed formula, Nutramigen. Utilizing tricine-SDS-polyacrylamide gel electrophoresis followed by silver staining, the presence of two allergenic protein fractions, 18 kD and 20 kD, were detected in Nutramigen. This method was not able to separate distinct protein fractions in the diffuse staining seen in Profylac and Alimentum at the lower molecular weights. Exten-

sively hydrolyzed formulae are generated from the enzymatic digestion of the casein or whey fractions of cow milk. Although most of the residual peptides are less than 1500 kD, some larger peptides may escape digestion and lot-to-lot variability may occur (8, 18).

Apparent specific-IgE antibodies to these peptides were detectable in 80% (12 out of 15) of cow milk allergic children tested, regardless of their clinical reactivity or SPT positivity to Nutramigen. Four out of five children in each group had serum specific-IgE to these protein fractions. Since only one group of children had positive oral challenges to Nutramigen, the presence of these immunologically recognizable peptides does not correlate with clinical hypersensitivity.

These results provide another example of the limitations of *in vitro* tests in predicting food allergic reactions in the clinical setting (19). In a child with IgE-mediated cow milk allergy, a negative SPT to an extensively hydrolyzed cow milk formula indicates that it is extremely unlikely that the child will experience an IgE-mediated reaction to the hydrolyzed formula. In children with a history of severe IgE-mediated reactions to cow milk and a positive SPT to the hydrolysate formula, it is still unlikely that the child will experience an allergic reaction. Nevertheless, such a child should receive his/her first dose of the formula in a medical setting so that appropriate medical therapy can be administered in the rare case of an allergic reaction, as recommended in the 1993 ESPACI Position Paper (18).

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